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Intracellular Protein and DNA Dynamics in Competent *Bacillus subtilis* Cells

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Summary

We have found that two DNA repair/recombination proteins localize differentially to the cell poles in competent *Bacillus subtilis* cells. RecA protein colocalized with competence protein ComGA, and its polar localization largely depended on ComGA and ComK activity, while RecN oscillated between the poles in a minute time frame, independent of any competence factor. Oscillation of RecN arrested upon addition of external DNA, suggesting that an interaction with incoming single-stranded (ss) DNA favors the localization of RecN at the pole containing the competence machinery. In agreement with this model, purified RecN protein showed ATP-dependent binding to ssDNA. Addition of DNA resulted in the formation of RecA threads emanating from the competence machinery. Our data show that in competent bacteria there exists a specifically positioned and dynamic ssDNA binding apparatus that accepts ssDNA taken up through the polar competence machinery and processes ssDNA for recombination with chromosomal DNA via extended RecA filaments.

Introduction

Competence refers to the physiological state in which certain bacteria take up DNA from the environment for intracellular recombination with their own DNA (Dubnau, 1999). This state is found in species from a broad spectrum of bacterial branches, and it is clinically relevant because several human pathogens are naturally competent and can acquire drug resistances via this pathway.

Bacillus subtilis is a Gram-positive soil bacterium that is investigated as a model organism in several aspects, mostly because at the transition between exponential growth and stationary phase, it can choose between three states, stationary growth, sporulation (development into an enduring spore), and competence. Interestingly, while under conditions favoring sporulation, a maximum of 80% of the cells undergo this developmental process, only up to 10% of the cells become competent under competence-stimulating conditions. Competence is induced through secreted peptide factors, which trigger a sophisticated regulatory system that ultimately leads to the synthesis of the ComK master transcription regulator, which in turn activates all necessary competence proteins. Uptake of DNA occurs through binding of double-stranded DNA by the membrane bound ComEA protein (Provvedi and Dubnau, 1999) but also requires the products of the *comG* operon, which appear to form a pilus-like structure (Chung and Dubnau, 1998). This multiprotein assembly could be required to channel DNA through the cell wall to ComEA. ComGA protein is involved in the assembly of the pseudopilus and is essential for the repression of growth and cell division during competence (Haijema et al., 2001). After binding, the DNA is cleaved into shorter fragments by the NucA endonuclease (Provvedi et al., 2001) and is converted to single-stranded (ss) DNA, which is taken up into the cytosol through the ComEC membrane permease (Chen and Dubnau, 2004). Uptake of DNA is thought to be energized by the ComFA ATPase (Londono-Vallejo and Dubnau, 1994). Only one DNA strand enters the cytosol, and the other is degraded and its cleavage products released into the medium (Chen and Dubnau, 2004). Recent single-molecule experiments have shown that the competence machinery is a strong motor that processively pulls DNA into the cell, without major pausing. The experiments supported the model that DNA is first reversibly bound by ComEA, followed by linear uptake, which appears to be driven or energized by the proton-motive force across the cell membrane (Maier et al., 2004). Interestingly, this competence machinery has recently been shown to be located at one of the cell poles (Hahn et al., 2005 [this issue of *Cell*]), suggesting that DNA uptake occurs at a single specific site in competent cells.

Within the cell, the imported DNA is used for homologous recombination with the chromosome, leading to the transformation of the cell, in case the acquired DNA contains novel or altered genetic information. Central to DNA recombination is the ssDNA binding ATPase RecA, which introduces ssDNA into a homologous DNA duplex, mediates DNA strand exchange, and opens up the avenue to the formation of Holliday junctions (crossovers) between homologous DNA duplexes (Cox, 2003; Kowalczykowski et al., 1994). Two pathways are thought to be required for efficient binding of RecA to ssDNA: one dependent on the RecBCD helicase/exonuclease complex (AddAB in *B. subtilis*) (Arnold and Kowalczykowski, 2000) and an alternative pathway driven by the RecN, RecO, RecR, and RecF proteins (Bork et al., 2001; Webb et al., 1997a). While the biochemical function of RecN, an ATPase and member of the SMC protein family, is quite unclear, recent data suggest that RecO and RecR facilitate loading of RecA to ssDNA, and RecF is thought to limit the extent of RecA binding (Bork et al., 2001; Kantake et al., 2002; Morimatsu and Kowalczykowski, 2003). The RuvABC complex (RuvAB/RecU in *B. subtilis*) resolves Holiday junctions, such that the exchange of DNA on the chromosome is terminated (Ayora et al., 2004; Cromie et al., 2001). Interestingly, it has recently been shown that RecN forms repair centers, to which first RecO and later RecF are recruited, on the nucleoids when DNA double-strand breaks (DSBs) occur (Kidane et al.,

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2004). These repair centers were irregular in the absence of RecN, suggesting that RecN plays a role as an initial DSB repair protein.

Because it is quite unknown which events occur within competent cells, and where they occur, we investigated the localization and mode of action of several proteins involved in DNA recombination, among them RecN and RecA. Both proteins localized to one cell pole but in distinct manners. RecA colocalized with the ComGA protein at one cell pole, exclusively in the 15%–20% of cells that were competent, while RecN oscillated between both cell poles, overlapping with but not strictly dependent on ComK activity. Because RecN is required for high efficiency of competence, our results suggest that RecN and RecA constitute or are a part of a protein assembly that accepts incoming ssDNA at a cell pole, where it is prepared for recombination with the chromosome.

Results

RecA Localizes to the Cell Poles in Competent Cells

We have recently found that defined DNA DSB repair centers (RCs) are formed in *Bacillus subtilis* cells upon induction of DSBs. RecN protein is the first factor that was found to assemble at a DSB on the nucleoids, while at a later time point, RecO and RecF proteins are recruited into the RCs (Kidane et al., 2004). We wished to investigate if recombination events can be visualized in competent cells. To this end, we created an N-terminal GFP fusion to RecA, which fully complemented the DNA repair defect of a *recA* deletion (data not shown). Strikingly, bright fluorescent foci were present in 19% of cells grown to competence (with 600 cells analyzed, Figure 1B), in about twice as many cells that usually become competent at the transition to stationary phase (Dubnau, 1999). Contrarily, GFP-RecA was never found at the cell pole in exponentially growing cells but specifically localized throughout the nucleoids (Figure 1A). A single GFP-RecA focus was found at or very close to one cell pole in 86% of the cells carrying a defined GFP-RecA signal, or at various positions within the cell in 12% of the cells grown to competence (Figure 1B). One percent of the cells contained elongated, needle-like GFP-RecA structures, pointing away from one cell pole (data not shown). Less than 1% of the cells contained two GFP-RecA foci. To investigate if RecA localization is static or dynamic, we performed timelapse microscopy. In general, GFP-RecA foci remained rather stationary at a single cell pole (Figure 2A, in 86% of the cells, with about 200 cells monitored). However, in 10% of the cells containing GFP-RecA foci, these were found to move within the cell, still being retained rather close to a cell pole (Figures 2A, left cell and 2B, left cell), while in 4% of the cells, a GFP-RecA focus moved from one pole to the other. The occasional movement of GFP-RecA foci was rather gradual (Figure 2B, right cell); in only one case did we observe that a focus moved from one pole to the other within a 1 min time interval. In the righthand cell in Figure 2B, the GFP-RecA focus splits between 4 and 12 min, and between 14 and 21 min, to unite back into a single focus, which was rarely but reproducibly observed. Thus, RecA shows dynamic localization in a subset of cells grown to competence.

To address the question of whether the polar localization of RecA depends on competence factors, we moved the GFP-RecA fusion into a *comK* deletion strain that does not express any competence factors. Contrarily to wild-type cells, GFP-RecA foci formed in only 1% of the cells grown to competence (data not shown, see Figure 1A), showing that GFP-RecA foci largely depend on the master competence factor ComK. This finding is in agreement with data showing that ComK directly mediates activation of transcription of *recA* during competence (Berka et al., 2002; Hamoen et al., 2001). GFP-RecA foci persisted for about 2 hr after reaching of full competence but started to dissipate after about 1 hr, showing that they are transient, largely competence-dependent assemblies.

Intriguingly, DNA uptake occurs at one cell pole in *B. subtilis* cells, at which the competence machinery is assembled (Hahn et al., 2005). As a marker for the uptake machinery, we employed ComGA-CFP (kind gift from J. Hahn and D. Dubnau). Seventeen percent of the cells grown to competence contained ComGA-CFP foci at a single cell pole, while 19% contained YFP-RecA foci. Strikingly, in 94% of the cells containing a ComGA-CFP focus at one cell pole, it was coincident with YFP-RecA, while in 6% of the cells, the foci were separate (Figure 3A). However, 3% of the cells contained a YFP-RecA focus but no ComGA-CFP focus (data not shown), while 1% of the cells contained a ComGA-CFP focus and lacked a YFP-RecA focus (Figure 3A), showing that although ComGA and RecA foci are predominantly colocalized, they are not necessarily present within the same cells. Thus, RecA is present at the cytosolic side of the competence machinery, most likely interacting with incoming ssDNA. Interestingly, RecA formed only foci in about 1% of competent cells in the absence of ComGA but was generally found on the nucleoids, as a much brighter signal compared to *comK* mutant or exponentially growing cells (Figure 1C, compare with 1A), showing that a direct link exists between RecA and the competence machinery, and that possibly, ComGA serves as an anchor for RecA or is required for the function of the factor anchoring RecA.

Interestingly, in cell doublets that have arisen through the last round of division of one cell, RecA was predominantly found at the old cell pole (Figure 3B, indicted by white triangles, in 74.6% of the cells, 725 cells analyzed) and less frequently at the new poles that have formed between the cells (gray triangles in Figure 3B, in 18.5% of the cells, and at other places in the cell in 6.9%). Timelapse experiments supported the observation that RecA has a bias for the old cell poles (Supplemental Data available with this article online).

RecN Oscillates between the Cell Poles within Minutes

To investigate if other repair proteins are also recruited to the competence machinery, we localized RecN-YFP in cells grown to competence. In contrast to exponentially growing cells, in which RecN is dispersed throughout the cells (Kidane et al., 2004), we found that RecN-YFP is predominantly found as a bright fluorescent

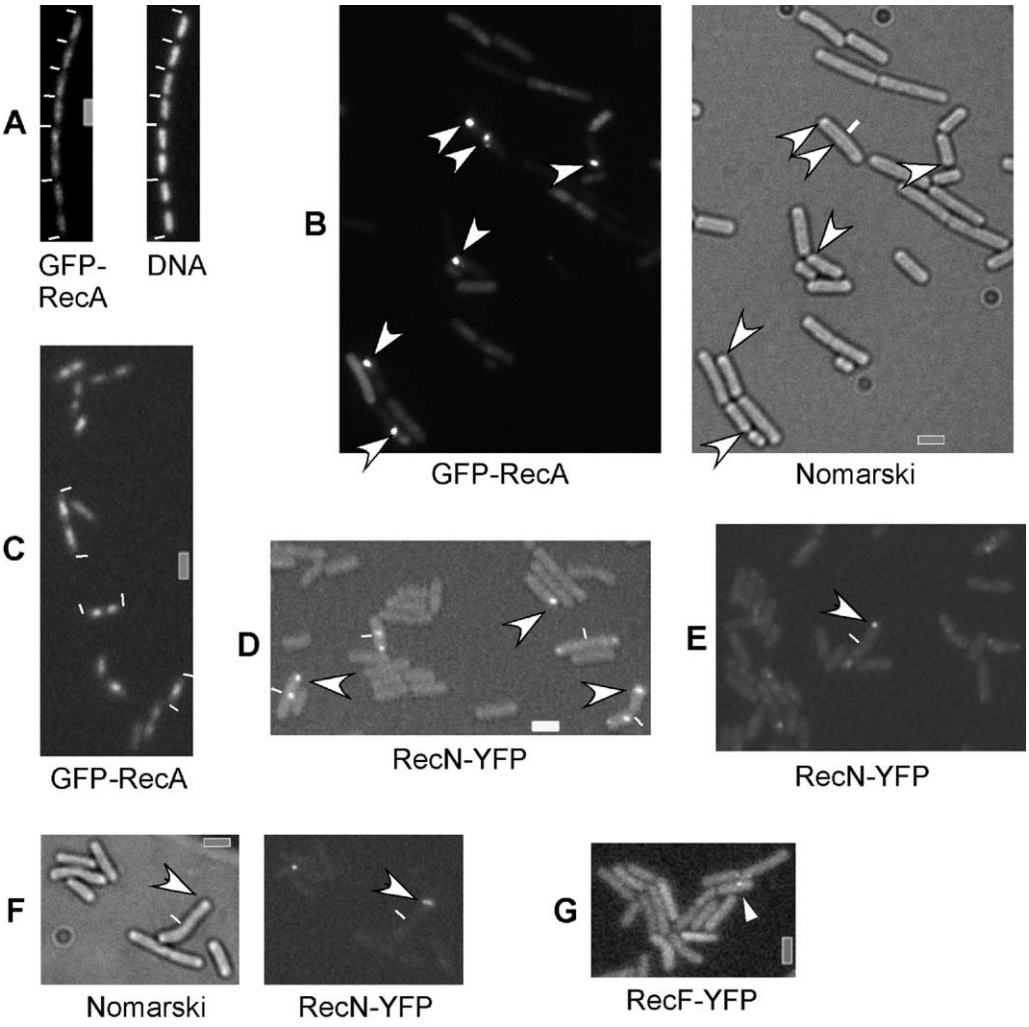

Figure 1. Fluorescence Microscopy of *Bacillus subtilis* Cells

(A) Cells growing exponentially or (B–G) grown to competence. (A–C) Cells expressing GFP-RecA, (D–F) cells expressing RecN-YFP, (G) cells expressing RecF-YFP. (A and B) Wild-type cells, (C) *comGA* mutant cells, (D) wild-type cells, combined acquisition of Nomarski and RecN-YFP fluorescence, (E) *comK* mutant cells, (F) *comGA* mutant cells. White lines indicate septa between cells, white arrowheads indicate polar RecN-YFP or GFP-RecA foci or a RecF-YFP focus in (G). Gray bars (white bar in [D]), 2 μm.

focus at a single cell pole (in 16% of >600 cells observed, Figure 1E) and rarely as a focus within the cell (in 1% of the cells) or close to the membrane away from the poles (in 3% of the cells). Rarely, two RecN-YFP foci could be seen (in 2% of the cells). Like RecA foci, RecN-YFP foci started to dissipate about 1 hr after reaching of the full state of competence. We employed timelapse microscopy to investigate if the foci have a fixed or flexible localization within cells grown to competence. Strikingly, RecN oscillated between both cells poles within 1–2 min intervals in about 90% of the cells containing RecN-YFP foci, as can be seen in Figures 2C–2F. Although the movement from one pole to the other lasted 1.6 s on average, and back movement 1.8 s, RecN-YFP remained at one pole much longer than at the other. Average time of persistence at one pole was 4.4 s, while persistence at the opposite pole was 1.2 s (e.g., Figure 2F). Moreover, RecN foci were frequently found to move away from one pole, and back to the pole (Figure 2D, between 10 and 15 min). The net result of this extended pausing at one pole was that on average, most RecN molecules were found at one preferred cell pole. Oscillation of RecN was observed in a decreasing number of cells at about 60 min after reaching of full competence, such that about 120 min after full competence, the few remaining RecN foci were completely static.

The movement of RecN-YFP from one pole to the other occurred in three different patterns: between most time intervals, it was straight between both poles, without any apparent intermediate step (e.g., Figure 2C, between 3 and 4 and 13 and 14 min); however, in 32% of all movements monitored, RecN-YFP foci were found at positions between the cell poles, predominantly close to the cell center (Figure 2D, between 2 and 9 min and Figure 2E, between 4 and 7 min) but also at posi-

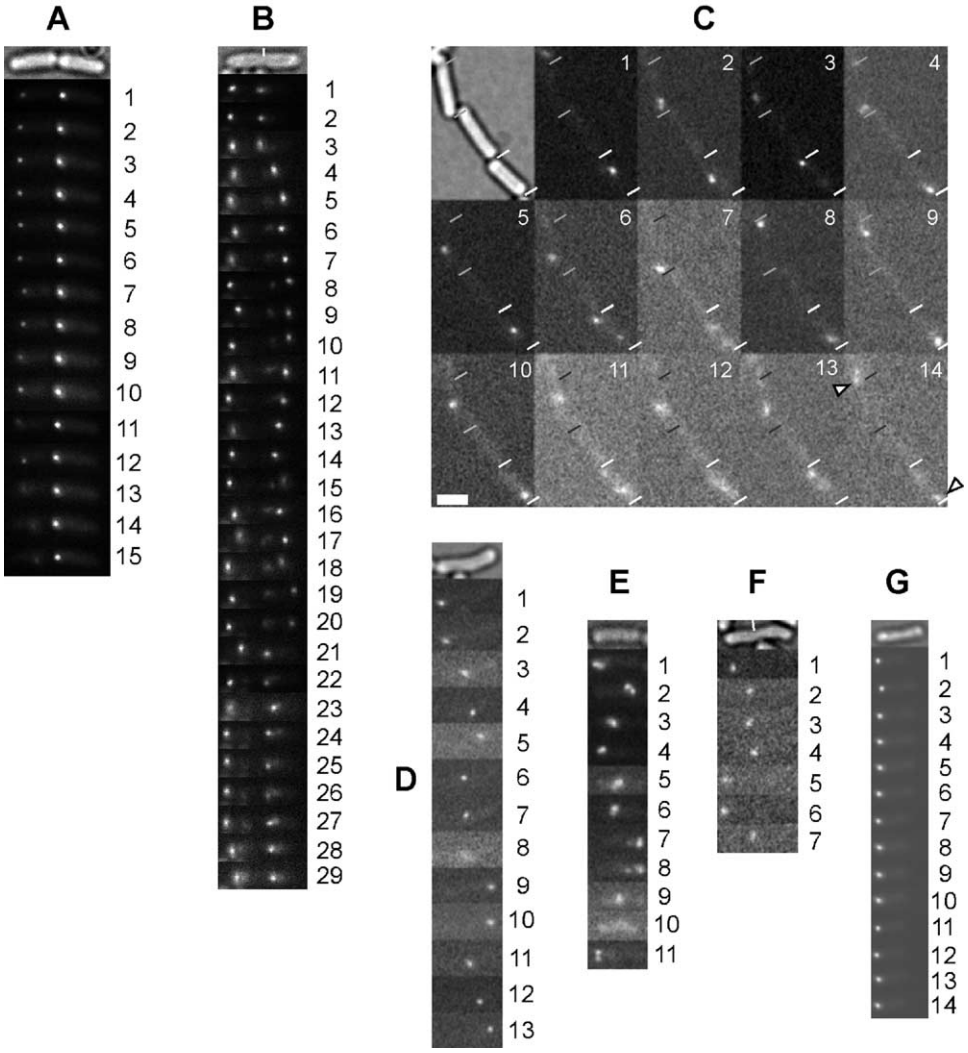

Figure 2. Timelapse Microscopy of *Bacillus subtilis* Cells Grown to Competence

(A and B) Cells expressing GFP-RecA, (C–G) cells expressing RecN-YFP. (A–E) Wild-type cells, (F) *comEC* mutant cells, to which DNA was added 10 min before timelapse acquisition, G) Wild-type cells, to which DNA was added 10 min before timelapse acquisition. Numbers indicate image intervals in minutes. White lines (or gray in 2C) indicate septa and ends of cells. Arrowheads indicate the position of RecN-YFP foci at the end of the experiment. All images are scaled equally; white bar in (C) indicates 2 μm.

tions off center; additionally, as mentioned above, RecN foci left a pole for another site within the cell and returned to the same pole, instead of moving to the opposite pole (in 5% of all movements). These observations suggest that there is no fixed anchor for RecN at the poles, but that RecN can localize to different sites within the cell, albeit with a high bias toward the poles.

Occasionally, a RecN-YFP focus could be seen to split into two (usually unequally intensive) foci, adding up to the intensity of the original focus; however, both foci united into one after a short time (1–2 min) (Figure 2C, between 5 and 8 min and Figure 2E, between 6 and 9 min). Thus, RecN-YFP foci appear to consist of dynamic assemblies of RecN molecules that can split up into fractions containing different numbers of molecules, which are highly efficient in reforming the original single assembly.

To find out if the oscillation of RecN has any significance for competence, we tested the efficiency of transformation of *recN* mutant cells. Compared to wild-type cells, a *recN* deletion strain showed a 4- to 5-fold reduced rate of formation of transformants, showing that RecN is not necessary but is important for competence. We also moved a GFP-RecA fusion into *recN*-deleted cells. GFP-RecA formed polar foci in *recN* mutant cells grown to competence (data not shown), showing that RecN is not required for the proper localization of RecA.

Contrarily to RecA and RecN, RecO or RecF did not form foci at any cell pole (Figure 1G and data not shown). Instead, in a low number of cells (1.8%), RecF was found to form a single discrete focus on the nucleoids (Figure 1G). Thus, RecO and RecF are not part of the polar assembly of RecA, ComGA, and RecN,

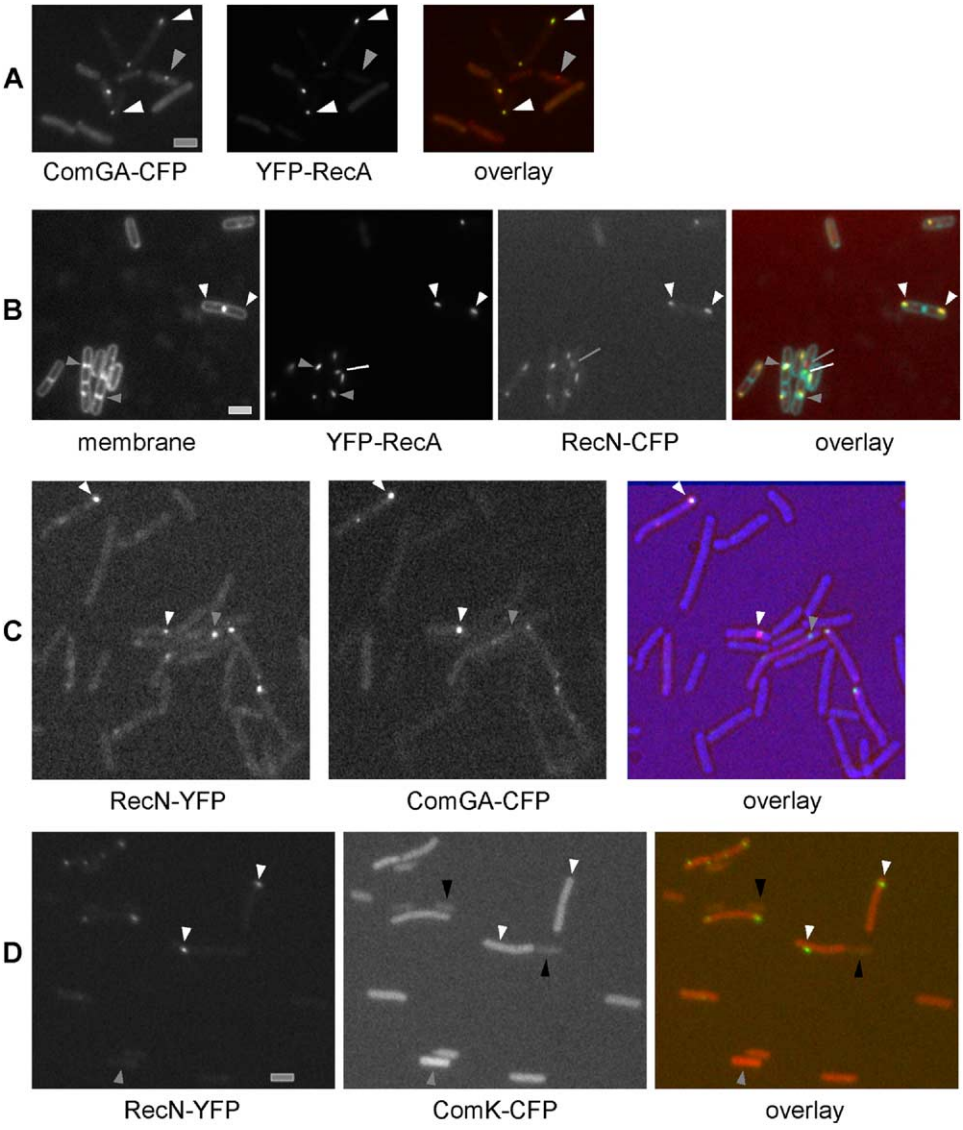

Figure 3. Fluorescence Microscopy of *Bacillus subtilis* Cells Grown to Competence Carrying Dual Labels

(A) Cells expressing ComGA-CFP (red in overlay) and YFP-RecA (green in overlay); white arrowheads indicate colocalization, gray arrowhead indicates a ComGA-CFP focus in the absence of a YFP-RecA focus. (B) Cells expressing RecN-CFP (red in overlay) and YFP-RecA (green in overlay); membrane stain in overlay is light blue, triangles indicate colocalized RecA and RecN, white triangles point out old poles, gray triangles new poles, and the white line indicates a polar YFP-RecA focus in a cell in which RecN-CFP is localized away from the pole, indicated by a gray line. (C) Cells expressing ComGA-CFP (red in overlay) and RecN-YFP (green in overlay); white arrowheads indicate colocalization, and the gray arrowhead indicates a cell containing a RecN-CFP focus but no ComGA focus. (D) Cells expressing ComK-CFP (red in overlay) and RecN-YFP (green in overlay); white arrowheads indicate cells with medium ComK signal containing a RecN-YFP focus, the gray arrowhead indicates a cell with high ComK activity lacking a RecN-YFP signal, and black arrowheads indicate cells with low ComK signal and no RecN-YFP signal. Gray bars, 2 μm.

but RecF might assemble at sites where recombination occurs between incoming DNA and the chromosome.

The Polar Localization of RecN Overlaps with RecA and ComGA but Is Not Dependent on Competence Factors

To test if RecN colocalizes with the polar RecA/ComGA assemblies, we created CFP and YFP fusion proteins and combined RecN-CFP or YFP fusions with YFP-RecA or ComGA-CFP constructs. In 84% of cells containing YFP-RecA foci, these colocalized with RecN-CFP foci (Figure 3B), while in the remaining cells, the signals were not coincident. In Figure 3B, a cell can be seen to contain a polar YFP-RecA signal and a more central RecN-CFP signal (indicated by white line and gray arrowhead, respectively), most likely representing a RecN assembly that is in the process of moving to the opposite cell pole. Similarly, Figure 3C shows that in most cells having a ComGA-CFP focus at a cell pole, the focus colocalizes with RecN-YFP (in 78% of the

cells) or is present at the other cell pole in 11% of the cells. However, 11% of ComGA-CFP containing cells did not contain a RecN-YFP focus, while RecN-YFP was also present in cells that did not contain a ComGA focus (in 3% of cells grown to competence, with 12% containing both ComGA-CFP and RecN-YFP foci, and 2% of the cells containing ComGA-CFP but no RecN-YFP foci). These data show that although RecN and ComGA mostly colocalize, they are not restricted to the same subpopulation of cells. To investigate further, if RecN-YFP-expressing cells indeed represent the competent subpopulation of cells, we moved a RecN-YFP fusion into a strain carrying a *cfp* gene whose expression is regulated by the ComK master competence transcriptional activator (Haijema et al., 2001). We categorized the cells to low/medium/high-fluorescence intensity, indicated by black, white, or gray triangles in Figure 3D. Interestingly, the polar localization of RecN was not strictly dependent on ComK activity. Although 45% of the RecN foci were present in cells that had high ComK levels, most cells containing RecN foci had an intermediate level of ComK activity (50% of the RecN-YFP-containing cells), and even 5% of the cells containing RecN foci had low ComK-CFP levels (Figure 3D). Conversely, cells with high ComK activity frequently lacked RecN-YFP foci (Figure 3D). Thus, RecN foci overlap with but are not restricted to cells expressing competence factors.

In agreement with these cytological data, we found that the polar localization of RecN is not dependent on competence per se. To test if RecN localization depends on any competence factors, the RecN-YFP fusion was moved into various mutant backgrounds. RecN-YFP foci were formed at a normal level in the absence of ComGA or of ComEC (Figure 1F and Figure 2F); however, fewer RecN-YFP foci were observed in *comK* mutant cells (in 16% of the cells compared with 22% of wild-type cells), and the foci were frequently weaker than in wild-type cells (compare Figure 1D with 1E). These data support the finding that ComK activity contributes to the formation of RecN-YFP foci but is not required for their assembly. Likewise, RecN-YFP showed normal oscillation between opposite cell poles in each of the mutant backgrounds (Figure 2F and data not shown).

Interaction of ComGA, RecA, and RecN at the Cell Pole

The colocalization of ComGA, RecN, and RecA led us to investigate if these proteins also physically interact at the cell pole. We employed FRET, using the CFP/YFP pairs created in this work. If a CFP molecule is very close to a YFP molecule (10 to 20 Å), emission of the CFP as a donor can lead to excitation of YFP. Employment of a filter that excites CFP and monitors YFP emission can reveal if FRET occurs between two fusion proteins. A Microimager allowed for simultaneous capture of the CFP and the FRET channels. RecN-YFP did not show any foci in the FRET channel (Figure 4A), while YFP-RecA showed a barely detectable signal (data not shown), so their contribution to the FRET signal was negligible or was subtracted from the actual FRET signal, respectively. ComGA-CFP signal intensity was 151 to 85 units (high and background values) on average in the FRET channel (140 cells analyzed) (Figure 4B), while average signal intensity was 188 to 82 units in cells expressing both ComGA-CFP and YFP-RecA (145 cells analyzed) (Figure 4C, upper panel). After subtraction of average signal intensity from the cytoplasm, average CFP/YFP FRET intensity for high values was 68, compared to 31 for cells expressing ComGA-CFP only, while the signal in the CFP channel was strongly reduced in the cells expressing the FRET pair versus the cells expressing solely ComGA-CFP (compare upper panel Figure 4C with 4B). In 90% of all cells analyzed, the FRET signal coincided with the YFP signal from the YFP-RecA fusion (Figure 4C, compare FRET with YFP). These data show that there is a strong interaction of ComGA and RecA at the cell pole in competent cells. On the other hand, the average signal intensity in cells expressing ComGA-CFP and RecN-YFP was 162 to 81 units, while the signal in the CFP channel did not change markedly compared to the ComGA-CFP-expressing strain (Figure 4D, upper panel, compare with Figure 4B). However, in 40% of the cells showing a ComGA-CFP signal (15% of all cells), the FRET signal was 20% higher compared with the control cells (Figure 4D, indicated by white arrowheads), while 3% of all cells showed a YFP signal but not FRET signal (Figure 4D, indicated by gray arrowhead). These data show that while many cells showed a FRET interaction between ComGA and RecN, a considerable number of cells did not have a FRET signal, in agreement with our findings that RecN oscillates between the cell poles and is thus not constantly present at the pole containing ComGA and is also present in cells lacking a ComGA-CFP focus.

Oscillation of RecN Is Influenced by Incoming DNA

To investigate if RecN might interact with exogenous DNA that is taken up into the cells by the polar competence assembly, we monitored the localization of RecA and RecN after addition of different types of DNA. Both proteins retained their polar localization; however, strikingly, RecN oscillation was only seen in a rapidly decreasing number of cells after addition of DNA. Ten minutes after incubation with ssDNA or with dsDNA (oligonucleotides, linear fragments, or circular plasmids were tested), only 4% of the cells containing RecN-YFP foci showed oscillation of RecN, while in the remaining cells, RecN remained at one cell pole (Figure 2G). Twenty minutes after addition of DNA, RecN movement was only observed in 2% of the cells, and in 1% after 30 min, when >50% of untreated cells contained oscillating RecN-YFP foci. When external DNA was added to cells simultaneously expressing RecN-YFP and ComGA-CFP, RecN-YFP was found to colocalize with ComGA-CFP in 96% of the cells (data not shown), showing that addition of DNA arrests RecN oscillation at the pole containing the competence machinery. To investigate if oscillation is arrested through incoming DNA, we performed timelapse experiments in a *comEC* mutant background. Intriguingly, the addition of external DNA did not have a considerable effect on oscillation of RecN in the absence of the ComEC uptake channel (78% of the cells containing RecN-YFP foci showed oscillation, Figure 2F), strongly suggesting that incoming DNA directly affects the localization dynamics of RecN.

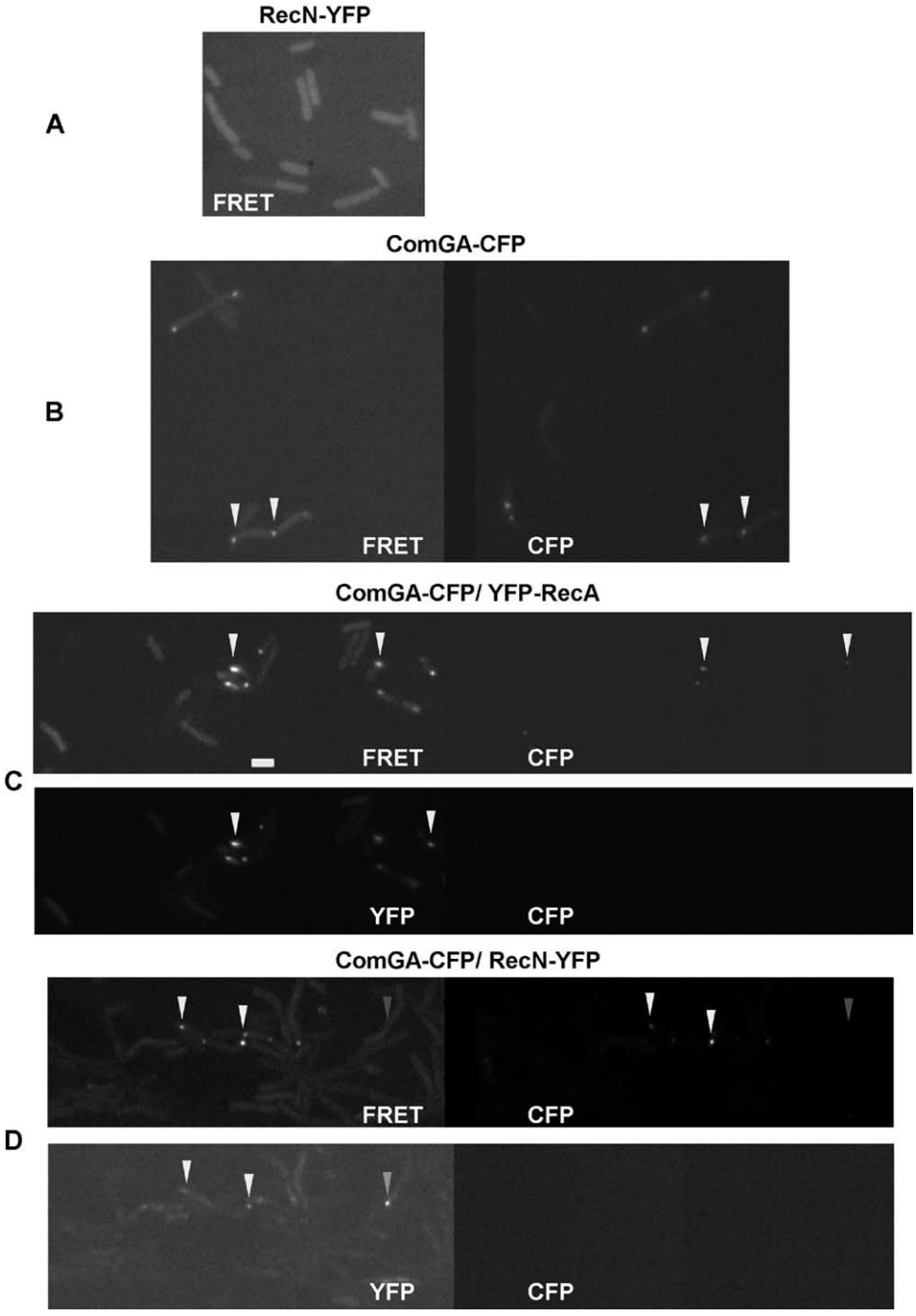

Figure 4. FRET Analysis of the Interaction of RecA, RecN, and ComGA

All cells were grown to competence. FRET/CFP: excitation with CFP exciter/emission with YFP filter (FRET) or with CFP filter, YFP/CFP: excitation with YFP emitter/emission with YFP or CFP filter. (A) Cells expressing RecN-YFP imaged using the FRET filter. (B) Cells expressing ComGA-CFP; white arrowheads indicate typical signal intensities. (C) Cells expressing ComGA-CFP and RecA-YFP, using filters as indicated; white arrowheads indicate typical signal intensities. (D) Cells expressing ComGA-CFP and RecN-YFP; white arrowheads indicate true FRET signals, and gray arrowhead indicates a YFP signal in the absence of a CFP signal. All FRET/CFP images have the same scaling for signal intensity and size; white bar, 2 μm.

RecN Is an ATP-Dependent ssDNA Binding Protein

We wished to gain further insight into the function of RecN. To study a possible interaction of RecN with nucleic acids, RecN protein was purified as a hexahistidine tag fusion after heterologous expression in *E. coli*. The protein was purified by a two-step column chromatography protocol to an apparent purity of >98% (data not shown). The protein interacted strongly with ssDNA in the presence but not in the absence of ATP. Figure 5 shows that a minimum of 100 ng of RecN was required

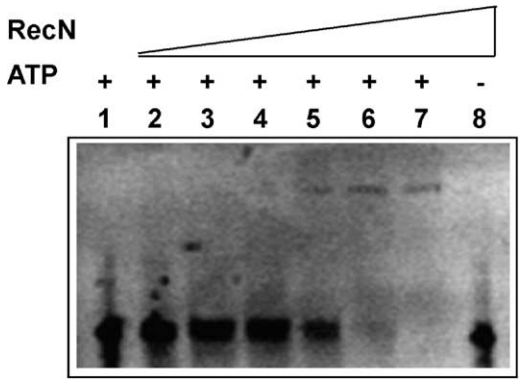

Figure 5. Ethidium-Bromide-Stained Gel-Shift Assays using Purified RecN Protein

60 base ssDNA (10 μM) was loaded into each lane, after incubation with (1) 0 μg, (2) 100 pg, (3) 1 ng, (4) 10 ng, (5) 100 ng, (6) 1 μg, (7) 10 μg, (8) 10 μg of RecN, in the presence or absence of 0.5 mM ATP, as indicated.

in the presence of ATP to achieve efficient binding to the ssDNA, whereas in the absence of ATP, no binding occurred even at an amount of 10 μg of RecN, which was able to completely shift the DNA in the presence of ATP. RecN bound to ssDNA in a nonspecific manner, but only a single shifted band was observed, suggesting a defined stoichiometry of RecN molecules binding to the ssDNA substrate. Interestingly, the shifted band reproducibly showed a weaker staining by ethidium bromide compared to the nonbound DNA, indicating that the bound DNA might be less accessible for the dye than the unbound ssDNA, or that RecN forms many different complexes with DNA that migrate heterogeneously through the gel. RecN did not show efficient binding to the double-stranded DNA tested (ssDNA from Figure 5 plus complementary strand) nor to a 500 bp DNA fragment or a 3 kb circular plasmid (data not shown), showing that RecN is an ATP-dependent ssDNA binding protein.

RecA Forms Transient Filamentous Structures Emanating from the Pole Containing the Competence Machinery after Addition of DNA

The fact that DNA uptake and binding of ssDNA occurs at a single cell pole raises the question of whether recombination of incoming ssDNA with the chromosome occurs at the cell pole or ssDNA is transported or guided to the corresponding sites on the chromosome. We therefore investigated if RecA might alter its pattern of localization in the presence of external DNA. Indeed, after addition of plasmid DNA, or of chromosomal *B. subtilis* DNA, RecA formed striking filamentous structures (we hereafter refer to as threads) within cells grown to competence (Figure 6A). Between 15 and 60 min after addition of DNA, up to 31% of all cells containing a GFP-RecA focus at a pole (thus the cells that are competent) showed extended threads (Figures 6A–6D), whereas 120 min after addition of DNA, GFP-RecA was found to generally localize to the nucleoids, similar to exponentially growing cells (albeit with much higher signal intensity compared with growing cells) (Figure 6E, compare with Figure 1A). The threads had highly variable lengths, from very short (0.25 μm) to extending almost throughout the entire cell (up to 2.2 μm), and could be straight or curved (Figures 6A–6D and 6G). Thus, the RecA threads are transient and highly heterogeneous structures. In 11% of the cells containing a GFP-RecA signal, a GFP-RecA focus at one pole was visible (indicated by white arrowheads), as well as a thread (indicated by gray arrowheads, Figures 6B–6D). In 90% of these cases, the threads connected to the polar foci, suggesting that they emanate from the polar RecA assemblies. To test this further, we visualized YFP-RecA threads in parallel with ComGA-CFP after addition of DNA. In all of the 20 cells observed that contained ComGA-CFP and a polar YFP-RecA focus as well as a thread, the ComGA-CFP focus colocalized with YFP-RecA, to which the thread connected in 17 cells (Figure 6G) (in 3 cells, the latter was close to the focus). Additionally, in 15 cells, a YFP-RecA thread connected with a ComGA-CFP focus (in the absence of a polar YFP-RecA focus), indicating that the RecA threads originate at the competence pole and extend into the cytosol. As a control, chromosomal DNA was added to a strain lacking the ComEC DNA uptake channel. Unlike wild-type cells, *comEC* mutant cells did not form any YFP-RecA threads, showing that the threads are indeed caused by incoming DNA (data not shown). To test if RecA threads are important for transformation, we took advantage of a RecA fusion to RFP that has a shorter linker than the functional YFP-RecA fusion (5 versus 10 aa, respectively) and that is the sole source of RecA expressed in the cells. Similar to the *recA* null strain, the transformation efficiency of the RFP-RecA strain was reduced about 1000-fold, contrarily to the strains expressing YFP-RecA or GFP-RecA whose transformation efficiency was indistinguishable from that of wild-type cells. Importantly, in cells grown to competence, RFP-RecA formed foci at the cells poles indistinguishable from cells expressing YFP-RecA but did not form any threads after addition of DNA (Figure 6F), most likely because the RFP tag interferes with the formation of RecA/ssDNA filaments. These experiments show that RecA threads are required for transformation and are most likely intermediates in recombination with the chromosome. Moreover, our results suggest that incoming ssDNA is brought to the corresponding site on the nucleoids for homologous recombination via RecA threads.

As a consequence of this, it should follow that chromosomal sites that are targets for recombination do not move to the cell pole but remain at their intracellular position after addition of homologous DNA. Indeed, we found that the conserved arrangement of the chromosome present in exponentially growing cells (Teleman et al., 1998; Webb et al., 1997b) is retained in competent cells in the presence or absence of external DNA and did not observe in a large number of cells analyzed any significant movement of chromosomal sites targeted for recombination to the cell pole (Supplemental Data).

Discussion

In this work, we show that a specifically localized intracellular machinery that accepts incoming DNA exists in

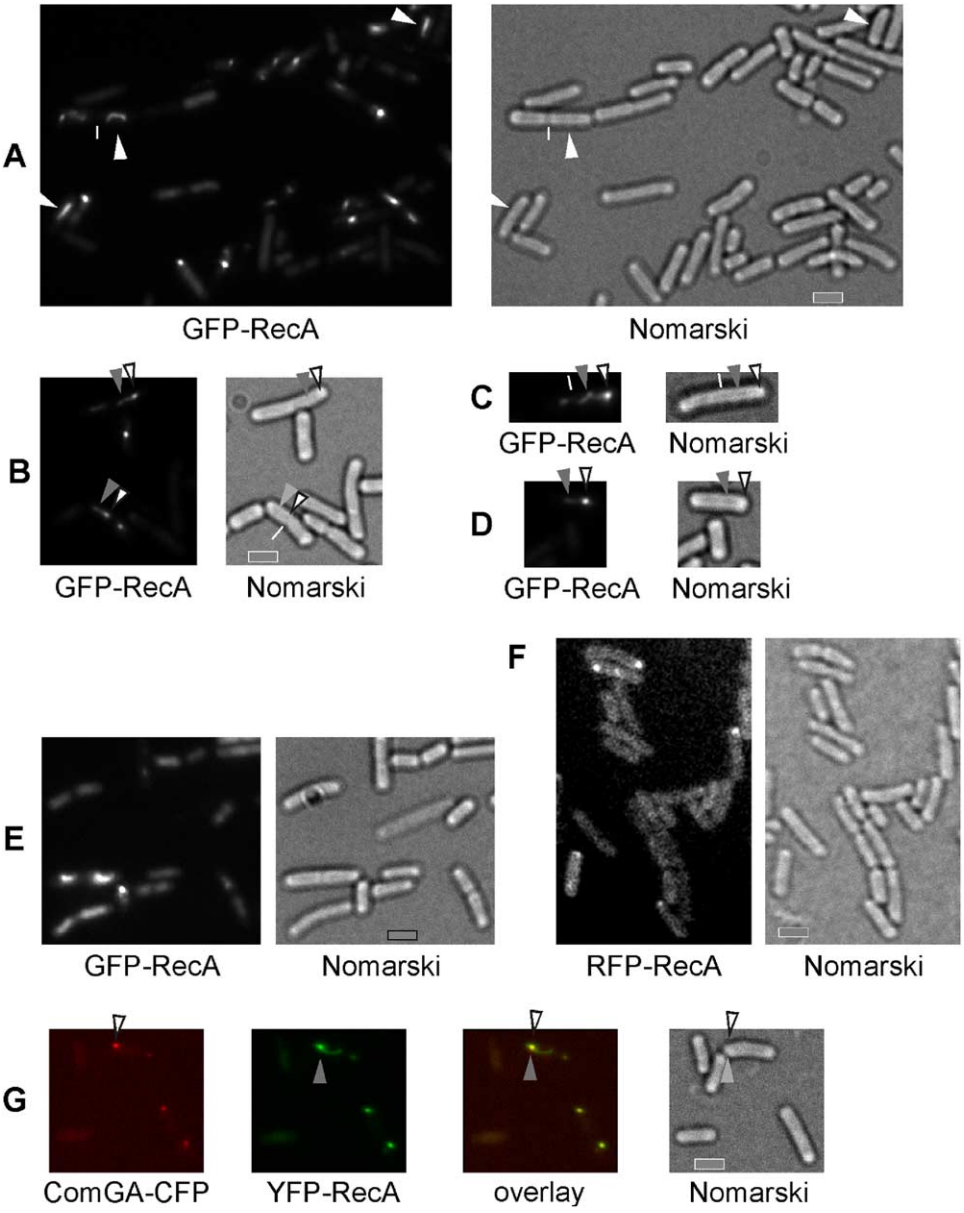

Figure 6. Fluorescence Microscopy of *Bacillus subtilis* Cells Grown to Competence to which Chromosomal DNA Has Been Added

(A–E) Cells expressing GFP-RecA; (A–D) 30–60 min after addition of DNA, (E) 180 min after addition of DNA. White arrowheads in (A) indicate RecA filaments within the cells, and polar RecA foci from which filaments emanate (indicated by gray arrowheads) in (B–E). (F) Cells expressing RFP-RecA to which chromosomal DNA has been added. The fusion is less bright than the GFP fusion and contains a shorter linker. (G) Cells expressing ComGA-CFP and YFP-RecA. White arrowhead indicates a polar ComGA-CFP focus, the gray arrowhead a YFP-RecA focus from which a filament emanates. Gray bars, 2 μm.

competent cells; the DNA is guided onto the nucleoids for homologous recombination with the chromosomal DNA, which retains its conserved pattern of subcellular localization. In agreement with recent results showing that DNA is taken up preferentially at one cell pole in competent *B. subtilis* cells (Hahn et al., 2005), we found that incoming DNA is welcomed at this site by at least two DNA repair/recombination proteins. We found that the major DNA recombination factor, RecA, and, to a lesser extent, RecN, colocalize with ComGA, a component of the DNA uptake machinery, at one cell pole. RecA is an ATP-dependent ssDNA binding protein and forms long nucleoprotein filaments in vitro, with six RecA monomers per turn and a pitch of 9.5 nm (Cox, 2003; Story et al., 1992; Xing and Bell, 2004). RecA introduces ssDNA into a homologous DNA duplex and mediates exchange of the homologous strands, such that a D loop is formed that is used for pairing with the remaining strand of the other duplex. This way, Holliday junctions and the potential for crossovers are set up between homologous duplices, aided by additional recombination proteins (Cromie et al., 2001; Haber, 2000). In agreement with recent findings from Sanchez and Alonso (2005), we found that like RecA, RecN is an ATP-

dependent ssDNA binding protein, suggesting that RecN also interacts with incoming DNA at the cell pole. However, most likely, RecN has a drastically different mode of ssDNA binding compared with RecA. RecN belongs to the SMC protein family, whose members are key players in various chromosome dynamics, such as chromosome condensation and cohesion, or DNA DSB repair (Hirano, 2002). SMC proteins consist of dimers that form a symmetrical structure with two long coiled-coil arms and a central hinge region. At the end of each arm is an ATPase head domain. Head domains can dimerize, leading to the formation of a closed ring structure. Bacterial SMC and cohesin SMC appear to bind to dsDNA by embracing DNA as a ring, which holds sister chromosomes together or possibly connects DNA loops (Gruber et al., 2003; Hirano and Hirano, 2002; Volkov et al., 2003). Interestingly, RecN is the first member of the SMC proteins shown to bind to ssDNA but not to dsDNA, the molecular basis of which will be interesting to investigate. Based on our finding that RecN binds to ssDNA and forms large nucleoprotein assemblies in cells having DSBs (Kidane et al., 2004), we propose that RecN may protect incoming DNA from degradation or functions as a ssDNA chaperone to prevent the formation of secondary structures. This agrees with our finding that RecN is not essential for transformation but confers an important function for the efficiency of this process. Further support for the notion that RecA and RecN interact with incoming ssDNA comes from our FRET studies that showed a strong interaction of ComGA and RecA and a weaker though still significant interaction of ComGA and RecN.

Curiously, RecN did not behave as a true competence factor. Contrarily to RecA, RecN also localized specifically in many noncompetent cells and oscillated between both cell poles. RecN assemblies were able to traverse up to 3 μm in 60 s, without apparent disassembly in between the poles, contrarily to the MinD protein, which does disassemble in between pole to pole oscillation (Raskin and de Boer, 1999) but forms extended helical structures (Shih et al., 2003). Also in contrast to MinD, the oscillation of RecN was asymmetric, with RecN staying at the competence poles for longer periods and with movements away from the pole and back, without reaching the other pole. Occasionally, however, RecN foci split into two discrete foci, showing that the assemblies are not static entities. RecN has been shown to form multimers in solution and to form large protein and nucleoprotein complexes after induction of DNA DSBs (Kidane et al., 2004), which could be the basis for the formation of the dynamic assemblies during competence. It remains to be investigated if oscillation of RecN requires its putative ATPase activity (SMC proteins usually have low ATPase activity [Hirano, 2002]) and which other factors might influence this intriguing property. Interestingly, addition of external DNA arrested the dynamic movement of RecN such that it preferentially came to rest at the pole containing the competence machinery, the site at which DNA enters. This suggests that RecN screens both cell poles for incoming DNA, which it binds upon entry to facilitate downstream processes. Our findings that RecN foci are not present in all competent cells and overlap with but do not depend on high ComK activity suggest that RecN presents another bottleneck toward genetic transformation, in that only those cells are fully competent that express both RecN foci and the competence machinery.

However, not all proteins involved in DNA recombination form polar foci in cells grown to competence. Neither RecF nor RecO foci were found at any cell pole. The finding that, occasionally, RecF protein forms foci on the nucleoids in cells grown to competence suggests that the incoming DNA might be brought to the sites on the nucleoids for recombination (where RecF might accumulate, as it does during repair of DNA DSBs [Kidane et al., 2004]), rather than the case that the chromosomal DNA might be brought to the cell pole. This idea is supported by two findings. First, we did not observe any movement of defined regions on the chromosomes to the cell poles upon addition of DNA homologous to these regions. The arrangement of the chromosomes appeared to be relatively unaltered during transformation with homologous DNA. Secondly, we found that RecA, upon addition of DNA, forms striking filamentous structures, which emanate from the pole containing the competence machinery, and extend into the cytosol. These RecA threads were highly heterogeneous in their length and appearance, depended on DNA uptake, and appeared to be required for recombination. We favor the view that they represent dynamic RecA/ssDNA nucleofilaments that scan the chromosomes for the homologous duplex and thus bring incoming DNA to the corresponding site on the nucleoids. This is feasible because the DNA uptake motor in *B. subtilis* can easily internalize DNA with a contour length of 7 μm within 2 min without pausing (corresponding to 80 bp/s) (Maier et al., 2004). RecA nucleofilaments have a similar axial rise per nucleotide as DNA (about 3 Å) (Story et al., 1992), so the longest filaments (2.2 μm) observed in this study would contain about 7000 nucleotides, while competent *B. subtilis* cells are able to continuously take up 10,000 bp within 2 min.

The fact that the ComEC complex takes up ssDNA, rather than dsDNA, and that RecA is present at the site of DNA uptake suggests that it is ssDNA that is used for recombination with the chromosome, rather than dsDNA built up from incoming ssDNA fragments. These findings strongly support the interesting concept that ssDNA is directly used for strand exchange and D loop formation, rather than recombination with dsDNA built up from the incoming ssDNA (Dubnau, 1999; Dubnau and Cirigliano, 1973). The D loop would need to be degraded, and a heteroduplex forms if the incoming DNA differs from the chromosomal DNA in some places. In this case, a transformant arises during the next round of replication and after segregation of the heteroduplex. If a homologous fragment containing a heterologous insertion (such as a resistance gene) is introduced, this would require the formation of a loop within the incoming DNA and subsequent repair, or replication, of the double-loop structure. Alternatively, a DSB might occur, followed by DSB repair. Thus, transformation appears to require heteroduplex formation, replication, and single-strand gap filling, rather than the formation of true crossovers. It will be interesting to investigate if additional recombination/repair proteins can be visualized

during transformation, which might provide additional support for the above model.

In toto, the above results suggest that during transformation, DNA that enters the cells at one cell pole is received and possibly protected by RecN protein, and by RecA protein, which is loaded onto the ssDNA, apparently without any need for RecO (contrarily to recombination and DSB repair [Bork et al., 2001]). Next, RecA forms long and apparently dynamic threads extending away from the site of uptake, which may scan the chromosome for the homologous duplex. Strand exchange appears to occur on the chromosomes that generally keep their arrangement within the nucleoids. Thus, the specifically localized DNA uptake machinery extends into the cytosol, such that a complex competence machinery provides a defined vectoral path for DNA from one cell pole to a site on the chromosome, showing that bacterial transformation is a highly organized and ordered process.

Experimental Procedures

Growth Conditions

Escherichia coli XL1-Blue (Stratagene) and *B. subtilis* were grown in Luria Bertani (LB) rich medium supplemented with 50 μg/ml ampicillin where appropriate. All the strains in this study are listed in the Supplemental Data. For microscopy, cells were grown in $S7_{50}$ medium. Competent cultures were grown as described previously (Dubnau and Davidoff-Abelson, 1971).

Construction Vectors and Strains

To create an N-terminal fusion of RecA with GFP for double crossover integration into the chromosome, the *recA* gene was amplified by PCR and was cloned in to ApaI and EcoRI sites of pSG1729 (Lewis and Marston, 1999). *B. subtilis* PY79 wild-type cells were transformed with the resulting plasmid (selecting for spectinomycin [spec]), which stably integrated into the amylase locus on the chromosome, establishing strain DK37. The GFP-RecA fusion is fully active because the original *recA* gene can be deleted without loss of DNA damage resistance or loss of viability (*recA* mutant cells grow much slower than wild-type cells and have a markedly reduced plating efficiency [Sciochetti et al., 2001]). Expression of *gfp-recA* was driven by addition of 0.5% xylose. Lowering the expression level through addition of 0.05% xylose still fully supported RecA function and showed similar localization patterns to those in higher xylose levels, ruling out artifacts through RecA expression levels (data not shown). A YFP fusion of RecA was constructed from *gfpmut2* by site directed mutagenesis, generating strain DK38. The RFP-RecA fusion was constructed by cloning N-terminal 550 bp of RecA into plasmid pSG1164 containing RFP, which was integrated at the *recA* locus by single crossover. To move the YFP-RecA fusion into various other backgrounds, strain DK38 was transformed with chromosomal DNA of strains containing a *recN-yfp* or a *comGA-cfp* fusion or with chromosomal DNA of *comK* or *comGA* mutant cells (gifts from D. Dubnau), generating strains DK42, DK64, DK66 or DK67, respectively. A strain carrying RecN-YFP and an origin tag was constructed by transformation of strain PG25 (*lacI-cfp*, *lacO* cassette at 359°) (Mascarenhas et al., 2002) with chromosomal DNA from DK01, resulting in strain PG33. Strain DK01 was transformed with chromosomal DNA from *comGA, comK*, or *comEC* mutant strains, resulting in strains PG30, PG31, or PG32, respectively. To create a strain in which ComK activity can be visualized in cells expressing RecN-YFP, strain DK01 was transformed with chromosomal DNA from a strain carrying a fusion of the first five codons of *comK* (which is autoinduced) to *cfp* (Haijema et al., 2001), generating strain PG34.

Image Acquisition

Fluorescence microscopy was performed on an Olympus AX70 microscope. Cells were mounted on agarose gel pads containing $S7_{50}$ growth medium on object slides. Images were acquired with a digital CCD camera; signal intensities and cell length were measured using the Metamorph 5.0 program (Universal Imaging Corp.). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; final concentration 0.2 ng/ml), and membranes were stained with FM4-64 (final concentration 1 nM). FRET was performed using a CFP excitater/dichroic filter cube (or a YFP excitater/dichroic filter cube to visualize YFP fluorescence) and a MultiSpec microimager (Visitron Systems, Germany) equipped with a beam splitter and CFP and YFP emission cubes. All images were acquired using 200 ms exposure time. FRET values were calculated by measuring fluorescence intensity in individual cells from different fields of cells expressing no GFP fusion (control) or expressing different combinations of GFP fusions. True FRET intensity was calculated by subtracting average background and intracellular fluorescence levels from wild-type cells devoid of any GFP fusion from corresponding average FRET fluorescence in strains carrying YFP/CFP fusion.

Supplemental Data

Supplemental Data include text, two figures, and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/122/1/73/DC1/.

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